

Effect of extrahypophysial gonadotrophins on the mammary glands of hypo- physectomized rats injected with insulin¹

by

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In animals with intact pituitary gland, growth and differentiation of the mammary gland is stimulated by actions of ovarian hormones. The growth response of the mammary glands is considerably reduced or absent when hypophysectomized animals are injected with ovarian steroids. This failure of the mammary gland to respond to the stimulating actions of ovarian hormones may be due to the lack of hypophysial hormones with specific actions on the mammary gland tissues, to alterations of the general metabolism following the ablation of the pituitary gland or to both. From the work of foremost LYONS and coworkers it is evident that the anterior hypophysial factors, prolactin and growth hormone, are involved in mammary gland growth. A direct action of prolactin on mammary gland tissues is indicated by experiments of e.g. LYONS, but so far little is known about the role played by the intermediary metabolism which is known to be influenced by prolactin and growth hormone. (Lit. see FOLLEY and MALPRESS, 1948; NELSON, 1952; JACOBSON, 1954; COWIE and FOLLEY, 1955; LYONS, JOHNSON, COLE and LI, 1955; AHRÉN and JACOBSON, 1956).

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As shown by SALTER and BEST, 1953, and LAWRENCE, SALTER and BEST, 1954, the general metabolism of hypophysectomized rats supplied *ad libitum* with a carbohydrate rich diet is altered by daily administration of long acting insulin. The authors mentioned found, amongst other things, that voluntary food intake of such rats was increased, and body weight and length as well as nitrogen retention increased. Thus, treatment of hypophysectomized rats with insulin promoted changes favouring the establishment of growth processes. The growth of mammary glands under conditions similar to those prevailing in the experiments of BEST and coworkers was studied recently and it was observed that castrated, hypophysectomized rats injected with oestrone, progesterone and long acting insulin presented growth and differentiation of the mammary glands (ÅHRÉN and JACOBSON, 1956). These observations showed that mammary gland growth can be promoted in the absence of hypophysial hormones. Apparently, the treatment with insulin had increased the responsiveness of the mammary gland to the stimulating actions of the ovarian steroids injected.

One of the questions arising from the work of ÅHRÉN and JACOBSON, 1956, is: how does the mammary gland, under similar conditions, react upon endogenously produced ovarian hormones? The study of this problem is met with difficulties arising i.a. from the fact that the functional activity of the corpora lutea is dependent upon the luteotrophic action of prolactin, which, as mentioned above, presumably exerts a direct stimulating action upon the mammary gland as well.

In the present experiments ovarian activity was stimulated with pregnant mare's serum gonadotrophin (PMS) or human chorionic gonadotrophin (HCG). Observations made on the mammary glands of hypophysectomized rats treated with insulin and/or these gonadotrophins will be reported in the present communication.

Experimental

A total of 60 young female rats bred at our Institute were used. Their diet given *ad libitum* consisted of bread, dog biscuits, mixed grain and fresh milk supplemented by glucose during the period of injections.

Four groups of completely hypophysectomized rats treated as follows were studied:

- Group I* (10 rats): Injections of PMS.
Group II (13 rats): Injections of PMS and long-acting insulin.
Group III (4 rats): Injections of HCG.
Group IV (9 rats): Injections of HCG and long-acting insulin.

In addition observations were made on the mammary glands of incompletely hypophysectomized rats and of rats with intact pituitary gland after treatments as in groups I to IV.

As far as possible littermates were distributed equally amongst the groups mentioned.

Hypophysectomy was performed when the rats were 31 to 33 days old (mean body weight $64 \pm 1,3$ (56) g), that is before their vaginal orifice had opened. Remnants of hypophyseal tissue were always searched for at autopsy at $2.5 \times$ magnification and then by microscopic examination of serial sections through the hypophyseal capsule and adjacent tissues embedded in paraffin wax, cut at 10μ and stained with hematoxylin eosin. Remnants indicated below as "microscopic" consisted of small groups of atypical or dedifferentiated anterior lobe cells in contact with the pars tuberalis surrounding the pituitary stalk or isolated by connective tissue. Such remnants were never detected with the $2.5 \times$ magnification at autopsy. The hypophysectomy was regarded as complete only when the microscopic examination did not reveal any remnant.

For the injections of insulin, zinc insulin lente (Novo) containing 40 i.u. per ml was used. The dose of insulin injected once daily (at about 12 o'clock) subcutaneously was gradually increased according to the following scheme: 1 i.u. during 2 days, 2 and 4 i.u. during 4 days each and 6 and 8 i.u. during 6 days each. In a few exceptions 4 i.u. were given during 6 days. For details concerning the insulin treatment see ÅHRÉN and JACOBSON, 1956.

The preparation of PMS was Gestyl (Organon) 1000 i.u. per ampoule. As a rule 100 i.u. (0,1 ml of 1000 i.u. in 1.0 ml saline) were injected subcutaneously every 3rd or 4th day.

The preparation of HCG, Pregnyl (Organon) 1500 i.u. per ampoule, dissolved in 1.0 or 2.0 ml saline was injected subcutaneously in doses of 75 to 200 i.u. once daily. Administration of 400 i.u. was effected by injections of 200 i.u. twice daily. The injections of HCG as well as of PMS were begun either a few days before or after the start of the insulin treatment. Care was taken to inject the two preparations into different sites under the skin of the rats. Details about doses, length of treatment with the two gonadotrophic preparations and start as related to insulin injections are given under "results". The treatment with the preparations mentioned was instituted at about two months after hypophysectomy.

The functional activity of the ovaries was judged from the condition of the vaginal membrane, from the appearance of vaginal smears and, at autopsy, from the size of the uterine horns. The ovaries were removed

at autopsy, weighed, embedded in paraffin wax, cut serially at 10 μ , stained with hematoxylin eosin and examined microscopically.

Mammary glands of all rats were examined shortly before the beginning of the injections and at the end of the experiment. Corresponding glands from one and the same rat (usually the second thoracic glands) were compared with each other after they had been stained with gallo-cyanin chromalum and mounted in toto. Parts of a few glands were embedded in paraffin wax for microscopic examination. Technical details about the preparation of the mammary glands as well as the criteria used for the judgement of mammary gland growth may be found in previous work (JACOBSON, 1948). Figures 1-11 illustrate typical results. In the tables growth is indicated as + or ++, absence of growth as 0.

The body weight of the rats was taken at hypophysectomy, at removal of the first mammary gland and at short intervals during the period of injections. The adrenal glands were removed post mortem and weighed.

RESULTS

Group I

Injections of PMS. This group comprises 18 rats, 10 of which had been completely hypophysectomized, in 4 others small remnants of anterior lobe cells were found at microscopic control, 2 had remnants detected at $2.5 \times$ magnification and in another 2 rats the pituitary gland was left intact.

The experiments performed on the completely hypophysectomized rats are summarized in Table 1.

TABLE 1

Completely hypophysectomized female rats injected with PMS

Exp.	Body wt. at:		Doses of PMS *	Ovaries after last inj.		Growth of m. gl.
	first inj. g	exam. g		days	mg	
1	65	60	20	4	8.9	0
2	105	105	100 \times 2	5	37.7	0
3	75	75	100 \times 3	3	36.7	0
4	105	100	20; 200	6	34.8	0
5	115	110	100 \times 3	2	34.5	0
6	100	105	100 \times 3	4	23.7	0
7	65	65	100 \times 3	4	13.6	0
8	65	75	100 \times 3	6	8.3	0
9	100	90	100 \times 6	3	76.2	0
10	95	85	100 \times 8	1	61.3	(+)

* Injections given every 3rd day in exp. 3, 9 and 10, otherwise every 4th day.

The body weight of these rats, operated on when immature, had generally increased slightly during the two months elapsing between hypophysectomy and start of injections. During the experiments lasting from 4 to 22 days the body weight remained unchanged. As may be seen from table 1, the doses of PMS were fairly high and the weight of both ovaries, reduced to about 4 mg in uninjected rats hypophysectomized at the age of one month, was considerably increased in all but experiments 1 and 8. The rat of exp. 1 had received the lowest dose and that of exp. 8 was examined 14 days after the first, that is 6 days after the last, injection. Microscopic examination of the ovaries of exp. 8 revealed a regression of the luteinization found in the ovaries of the other rats. The number of follicles with antrum appeared limited in the enlarged ovaries showing mainly luteinized follicles and a luteinized interstitium. Ovulation did not seem to have occurred. The vaginal orifice opened in all but exp. 1 between the 6th and 10th day after the first injection, and the vaginal smears as well as the appearance of the uteri indicated oestrus. The adrenal glands appeared as atrophic as in untreated hypophysectomized rats. Their mean weight was $6,4 \pm 0,37$ (10) mg.

In experiments 1-8 lasting up to 14 days the mammary glands examined post mortem presented roughly the same picture as the corresponding glands removed before the injection. Indications of growth and differentiation were not found (Fig. 1). The glands examined 18 and 22 days after the beginning of injections of a total of 600 and 800 i.u., respectively (expts 9 and 10) remained also undeveloped, but presented ducts with increased diameter (Fig. 3 and 4) due, as confirmed microscopically in exp. 10, to an accumulation of secretion within the ducts. Because of the distension of the ducts it is difficult to decide whether some growth had occurred, but in exp. 10 this seemed to be true.

In 2 of 4 rats with remnants of hypophysial tissue revealed microscopically, the ovaries were somewhat more enlarged and the mammary glands showed slight growth of side buds and end buds. The remaining 2 rats reacted as the completely hypophysectomized animals of the present group. The 2 rats with large remnants and those with intact pituitary gland presented heavy ovaries (between 150 and 360 mg) with large follicles, bloodfollicles, corpora lutea and luteinized follicles and large interstitial cells.

The mammary glands were enlarged and the extensively developed alveoli could be clearly observed already at the dissection.

Group II

Injections of PMS and long-acting insulin. Of 23 rats subjected to this treatment 15 were completely hypophysectomized, 7 had microscopic remnants and in 1 rat the pituitary gland was left intact.

Data concerning experiments on 13 of the 15 completely hypophysectomized rats may be found in Table 2.

TABLE 2

Completely hypophysectomized female rats injected with PMS + insulin

Exp.	Body wt. at:		Period of insulin inj. *	Doses of PMS **	Ovaries after last inj.		Growth of m. gl.
	first inj.	exam.			days	mg	
	g	g	days	i.u.			
1	90	95	9	100	4	15.2	0
2	60	85	10	20	4	15.4	0
3	75	90	11	100 × 2	1	16.6	+
4	80	90	14	100 × 3	2	30.3	++
5	85	105	15	100 × 3	3	33.9	+++
6	75	110	16	20; 200	6	27.8	+++
7	85	105	16	100 × 3	2	35.8	+++
8	90	125	18	100 × 3	4	44.9	+++
9	90	115	18	20; 100; 200	2	40.3	+++
10	110	145	22	100 × 3	6	20.2	+
11	75	—	4	100 × 5	1	16.4 ***	0
12	90	95	7	100 × 6	1	57.8	+
13	95	105	9	100 × 6	3	22.3	+

* Injections of insulin begun 6 days *before* PMS in exp. 1-9, and at 8 days in exp. 10. In exp. 11-13 insulin treatment begun 9 days *after* the first injection of PMS.

** Injections given every 3rd day in expt 1, 4, 5, 11, 12, 13, otherwise every 4th day. Interval between 2nd and 3rd inj. in exp. 9, 6 days.

*** One ovary only.

Two of the 15 rats mentioned died shortly after the first injection of PMS, that is at the 8th day of insulin treatment. The body weight of all the 15 rats mentioned increased markedly during the period of injections. As indicated in the Table 2 the treatment with PMS was instituted either after or before the beginning of the insulin injections. The ovaries presented roughly the same

changes as those of the experiments given in Table 1 although follicular growth appeared, perhaps, more accentuated. Oestrus was indicated by breakdown of the vaginal membrane, by cornified cells in the vaginal smears and an enlargement of the uterine horns. These reactions were obtained in expts 4 to 13. In expts 2 and 3 the vaginal orifice remained closed. The rat of exp. 1 hypophysectomized at the age of 47 days (the only rat operated on later than at 33 days of age) had an open vagina before the experiment, but the vaginal smears indicated anoestrus. The mean weight of 8 ± 2.3 (13) mg indicates an atrophy of the adrenal glands.

Contrary to the findings in group I injected with PMS alone the mammary glands of the rats of expts 4 to 10 (Table 2) presented a distinct proliferation of side buds and end buds (Fig. 2). The diameter of the ducts often appeared enlarged. In these experiments the dose of insulin was 4 i.u. at the first injection of PMS and 6 and 8 i.u. daily at the end of expts 4, 5 and 6 to 10, respectively. Growth of the mammary glands was absent in the rats that had received only one injection of PMS (exp. 1 and 2) and in the rat (exp. 11) injected with insulin 1 and 2 i.u. during 2 days each. The rat (exp. 3) that died on the 5th day after the first injection of PMS presented slight growth of the mammary gland. Limited growth was observed in expts 12 and 13. Here the few side buds appeared elongated and thickened (Fig. 5 and 6). Since the insulin treatment was begun 9 days after PMS (expts 12 and 13) the dose of insulin had not been increased to more than 4 i.u. when the experiment was concluded.

In the 7 rats with microscopic remnants the reaction of the mammary glands was the same (2 rats) or slightly more extensive (5 rats) than in the completely hypophysectomized rats just mentioned. The ovaries and the mammary glands of the rat with intact pituitary gland presented the same hypertrophy as that found in group I in the rats with intact pituitary gland.

Group III

Injections of HCG were given to 9 rats, 4 of which had been completely hypophysectomized, in 4 others a small remnant of anterior lobe cells was found microscopically and in 1 rat a larger remnant had been left.

TABLE 3

Completely hypophysectomized female rats injected with HCG

Exp.	Body wt. at:		Doses of HCG *	Wt. of both ovaries mg	Wt. of both adrenals mg	Growth of m. gl.
	first inj. g	exam. g				
1	115	120	400 × 2 200 × 3	25.1	9.2	0
2	75	65	150 × 8	17.1	5.7	0
3	80	85	150 × 11	24.9	7.7	0
4	100	105	75 × 16	8.6	5.6	0

* Autopsy after last injection: exp. 1 at 4 days; exp. 2-4 at one day.

As may be seen from Table 3, summarizing experiments after complete hypophysectomy, the total amount of HCG administered was high. Except in exp. 4 the ovarian weight was markedly increased whilst the adrenal glands remained atrophic. The body weight was stable during the period of injections of 8 to 16 days. Microscopically the ovaries contained very few small follicles with granulosa cells, the dominating feature being a luteinization. Opening of the vagina and oestrus were found in experiments 3 and 4 after 8 and 10 days, respectively. Oestrus did not occur in expts 1 and 2, and changed towards metoestrus at the end of exp. 4. The mammary glands did not show growth of normal structures, but in expts 3 and 4 the ducts appeared slightly widened and the side buds seemed elongated (Fig. 7).

The mammary glands of the 4 rats with microscopic remnants showed no changes (1 rat) or slight augmentation of side buds and occasional end buds (3 rats). The reaction of the ovaries of these 4 rats was very similar to that observed in the experiments of Table 3. The one rat showing a macroscopic hypophysial remnant had larger ovaries (64.3 mg) with many follicles of different size, larger corpora lutea, luteinized follicles and enlarged interstitial cells. The mammary glands covering a greater area and showing end buds before the injections (Fig. 11) developed a number of alveoli in the central parts and a few side buds and many end buds in the periphery.

Group IV

Injections of HCG and long-acting insulin. 10 hypophysectomized rats, one of which had a macroscopic remnant, were injected with HCG in doses and during periods corresponding to those of group III. The experiments on the 9 completely hypophysectomized rats are summarized in Table 4.

TABLE 4

Completely hypophysectomized female rats injected with HCG + insulin

Exp.	Body wt. at:		Period of insulin inj. *	Doses of HCG**	Wt. of both ovaries	Growth of
	first inj. g	exam. g				
1	80	120	21	400 × 2 200 × 4	11.5	+
2	100	130	21	do	11.3	+
3	110	120	16	do	23.0	+
4	95	105	13	150 × 7	22.6	+
5	90	110	17	150 × 11	20.1	+
6	90	110	11	75 × 16	24.7	++
7	95	110	12	75 × 17	15.1	+
8	80	95	12	75 × 17	13.6	+
9	80	105	12	75 × 17	16.8	+

* Exp. 1-3 and 4, 5 injections of insulin begun 8 and 6 days, respectively, *before* HCG.
Exp. 6-9 injections of insulin begun 5 days *later* than HCG.

** Autopsy after the last injection: exp. 1, 2 at 8 days, exp. 3 at 3 days, exp. 4-9 at 1 day.

The period of insulin injections varied between 11 and 21 days. Thus, the daily doses of insulin had, at the end of the experiments, reached a level of 6 or 8 i.u. The body weight of all the 9 rats increased markedly during the period of treatment. The adrenal glands remained atrophic (mean weight 7.8 ± 0.36 (9)mg), but the ovaries had increased in weight and presented the same luteinization as those of group III. In all but expts 3 and 4 the vagina opened and the uterus appeared enlarged. In exp. 6 the opening occurred at 5 days after the first injection of HCG, in the remaining ones at 11 or 12 days. Oestrus was found in exp. 5, but expts 7 and 9 showed metoestrus and dioestrus.

The mammary glands removed after the injections showed an increase of the number of side buds, and in 5 rats end buds appeared. In expts 8 and 9 the diameter of the ducts seemed

enlarged (Fig. 8, 9, 10). The rat with a microscopic remnant of pituitary gland cells was treated as exp. 9, but during 21 days. The luteinization of the ovaries showed a beginning regress, but otherwise the result of the injections was the same as in the completely hypophysectomized rats of the present group. The weight of both adrenals was 8.2 mg.

DISCUSSION

The main result obtained in the present work was a stimulation of duct growth in the mammary glands of hypophysectomized rats injected with insulin in addition to PMS or HCG (group II and IV). The effect was dependent upon the dose of insulin. Daily injections of 6 to 8 i.u. seemed to be necessary. In view of the observations made by AHRÉN and JACOBSON, 1956, on castrated hypophysectomized rats injected with insulin, oestrone and progesterone, it seems tempting to assume that the stimulating effect of the gonadotrophin preparations was mediated by the ovaries. The absence of a development of alveoli would then have to be explained by a lack of progesterone secretion due to a failure of the gonadotrophins to exert a luteotrophic action on the ovaries. Supporting this assumption are reports of other investigators who failed, as confirmed in the present material, to produce ovulation and development of corpora lutea by injection of PMS or HCG into immature rats hypophysectomized a long time before the start of injections (LEONARD and SMITH, 1934; ROWLANDS and WILLIAMS, 1941; WILLIAMS, 1945; MORICARD, 1953). Additional evidence for a lack of a luteotrophic activity of PMS and HCG preparations was obtained by NELSON, 1952, studying the reaction of the mammary glands in hypophysectomized rats. Progesterone secretion and a progressive development of the mammary glands were observed by this investigator only when prolactin was given in addition to PMS and HCG. As mentioned in the introduction prolactin exerts a luteotrophic as well as a mammatrophic action in the rat. Since the extent of growth observed in the present experiments is apparently less than that described by e. g. LYONS, 1951, using prolactin in addition to FSH and ICSH, insulin does not seem to be able to replace the actions of prolactin. Further work is necessary to clarify this point, however.

In the hypophysectomized rats injected with PMS or HCG but not with insulin (groups I, and III) mammary gland growth was not obtained in spite of the fact that a stimulation of oestrogen secretion was indicated by the reactions of the vagina and uterus. This finding is in agreement with investigations showing that oestrogens do not promote mammary gland growth in the absence of anterior pituitary hormones and an exogenous supply of insulin (for lit. see introduction). After prolonged treatment an elongation of side buds and an increase of the diameter of the ducts were occasionally noticed. These changes might be due to a direct effect of the gonadotrophin preparation on the mammary gland tissues or to an effect promoted by androgenic agents, shown by PONSE, 1954, to be produced in the ovaries of hypophysectomized rats injected with PMS or HCG.

The mammary glands of rats with microscopic remnants of anterior hypophyseal tissues showed either the same changes as the corresponding completely hypophysectomized rats or a slight stimulation of growth. The adrenal glands of these rats were atrophic. The variations occurring in the results obtained from such rats emphasize the need of careful microscopic examination of the operation field.

The response of the reproductive organs of rats with larger remnants were similar to those described by others and confirmed in the present experiments on rats with intact pituitary gland. Some of the questions indicated above are objects of further investigations in this laboratory.

SUMMARY

The reaction of the mammary gland to high doses of PMS or HCG administered together with long-acting insulin was studied in female rats hypophysectomized before puberty and receiving the first injection after an interval of about two months.

Growth of ducts was obtained when insulin in doses of 6 to 8 i.u. per day was given together with PMS or HCG.

PMS or HCG given alone stimulated the ovaries to secrete oestrogens, but the mammary glands remained undeveloped. After prolonged treatment an abnormal elongation of side buds and a slight increase of the diameter of ducts was occasionally observed.

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COMMENTS TO PLATES I AND II

The figures show photographs of whole mount preparations of thoracic mammary glands stained with gallocyenin chromalum. The magnification is the same in all figures, x 8.5. The experimental procedures performed on the animal from which the gland was obtained are indicated below. Except for Fig. 11 all figures illustrate experiments on completely hypophysectomized rats.